Role of Heterologous and Homologous Glycoproteins in Phenotypic Mixing between Sendai Virus and Vesicular Stomatitis Virus

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Phenotypic mixing between Sendai virus and vesicular stomatitis virus (VSV) or the mutant VSV *ts045* was studied. Conditions were optimized for double infection, as shown by immunofluorescence microscopy. Virions from double-infected cells were separated by sequential velocity and isopycnic gradient centrifugations. Two types of particles with mixed protein compositions were found. One type was VSV particles with Sendai virus spikes, i.e., phenotypically mixed particles. A second type was Sendai virus-VSV associations, which in plaque assays also behaved as phenotypically mixed particles. The ratio of VSV G protein to Sendai virus glycoproteins on the cell surface was varied, using the VSV mutant *ts045* in double infections. Thus, different amounts of the VSV G protein were allowed to reach the cell surface at 32, 38, and 39°C in Sendai virus-infected cells. However, a fixed number of Sendai virus spikes was always found in the *ts045* virions. This represented 12 to 16% of the number of G proteins present in normal VSV. Furthermore, the yield of *ts045* virions was radically reduced during double infection when the temperature was raised to block G-protein transport to the cell surface, suggesting that the Sendai virus glycoproteins were not able to compensate for G protein in budding. These results emphasize the role of the G protein in VSV assembly.

Enveloped viruses can be considered as specialized membrane vesicles which transfer the viral genetic material from one cell to another. The genetic material of these viruses is first packaged in the form of a nucleocapsid in the cytoplasm. In the final stage of assembly, the nucleocapsid is wrapped within an envelope derived from the cell membrane. In this so-called budding process, only virally encoded proteins are accepted, while cellular proteins become excluded (1). A transmembrane interaction is required to bend the membrane into a bud and, further, into a vesicle (15). Experimental evidence was recently presented for Semliki Forest virus that its transmembrane spike protein interacts with the nucleocapsid (30). This interaction could explain the membrane bending and also how viral spikes are sorted into a budding virus and nonviral membrane proteins are excluded.

It has, however, been observed that enveloped viruses can adopt other viral glycoproteins in their membranes (for reviews, see references 31 and 34). This phenomenon is called phenotypic mixing. It has been documented by methods based on selective plaque assays or electron microscopy (2, 4, 5, 19, 32). A special case of phenotypically mixed particles is pseudotypes whose envelopes appear to contain solely foreign spikes. To what extent and by what mechanism viruses adopt foreign viral glycoproteins but not cellular proteins has remained an enigma.

In this work, we have quantitatively analyzed phenotypic mixing. Our approach was to quantitate how many Sendai virus spikes can be incorporated into a vesicular stomatitis virus (VSV) particle in double-infected cells. We exploited the VSV mutant *ts045* to vary the concentration of the G protein on the surface of double-infected cells and analyzed whether this affected the inclusion of Sendai virus spikes into VSV particles. The *ts045* mutant G protein is blocked in the endoplasmic reticulum at the nonpermissive temperature (10, 37), and very little G protein arrives at the cell surface.

Under these conditions, particle formation is reduced about 10-fold, and released particles are noninfectious (26). They contain only the carboxy-terminal fragment of the G protein, since the spikes are cleaved during and/or after budding (17). Our results show that at permissive and nonpermissive temperatures, foreign spikes are inserted into VSV *ts045* particles. The number of foreign spikes always corresponds to about 12 to 16% of the number of G proteins in wild-type (wt) VSV. We propose that VSV budding requires a full or a nearly full complement of homologous spikes, which cannot be replaced.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown in Eagle minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum and nonessential amino acids. Cells were used for infection after 4 days, when they reached confluency. wt VSV was the Indiana serotype. The mutant *ts045* was clone *ts045-6* (8). The Z strain of Sendai virus was generously provided by Robert Rydbeck, Department of Virology, Karolinska Institute, Stockholm, Sweden.

Conditions of double infection. All incubations were done in a humidified 5% CO₂ atmosphere in MEM supplemented with 0.1% bovine serum albumin and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.3). For double infection, cell monolayers were inoculated first with egg-grown Sendai virus (5 PFU per cell). Adsorption was for 1 h at 37°C. At 12 h after infection, the cells were washed and superinfected with either wt or mutant *ts045* VSV at a multiplicity of infection of 5. After 1 h of adsorption (32°C for *ts045*, 37°C for wt VSV), fresh medium was applied and the cells were incubated for 3 h at 37°C (wt VSV) or at 32, 38, and 39°C (*ts045*).

Metabolic labeling of virions. (i) Prelabeling. Cells infected with Sendai virus for 11 h were labeled with [³⁵S]methionine (Amersham Corp., Bucks, England) (200 µCi/ml; 0.75 µg of methionine per ml) for 1 h at 37°C. The cells were then

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superinfected with wt VSV or *ts045*, without removing the isotope. After 1 h of virus adsorption, the cells were washed, and the isotope-containing medium was replaced with normal medium. Incubation was continued for 3 h at the indicated temperatures, and virus-containing medium was harvested. This procedure resulted in a balance between the labeling of Sendai virus and wt VSV or *ts045* polypeptides in released virions and was used to localize virus-containing fractions after ultracentrifugation.

(ii) Continuous labeling. To uniformly label all viral polypeptides, Sendai virus-infected cells were incubated in MEM containing [³⁵S]methionine (200 µCi/ml; 1.5 µg of methionine per ml) for 12 h. Cells were then superinfected with wt VSV or *ts045*, without removing the label. After 1 h of adsorption, the cells were incubated with fresh isotope medium identical to the previous one. The temperature was adjusted as required, and virions were harvested at 4 h postinfection with VSV. In this procedure, the wt VSV or *ts045* polypeptides predominantly incorporated the radiolabel.

To label Sendai virus or VSV in single infections, cells were inoculated as described above and labeled with [³⁵S]methionine (100 µCi/ml; 0.75 µg of methionine per ml) for 10 to 16 or 1 to 4 h postinfection, respectively.

Purification of virions. Virions were purified by gradient centrifugation essentially as described previously (16). Medium from infected-cell cultures was clarified (5,000 × *g*, 20 min at 4°C) and layered on a 5 to 20% (wt/wt) linear sucrose gradient in 10 mM Tris (pH 7.3) containing 140 mM NaCl (TN buffer)–0.5 mM EDTA. Centrifugation was for 45 min at 20,000 rpm at 4°C in an SW28 or SW28.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Fractions were collected from the bottom by puncturing the tube and counted for ³⁵S radioactivity in a liquid scintillation counter.

Potassium tartrate gradient centrifugation was performed in a linear 16 to 22% (wt/wt) gradient in TN buffer, using a SW28 or SW28.1 rotor. After being spun at 25,000 rpm for 90 min at 4°C, fractions were collected and counted for ³⁵S radioactivity. Slow acceleration and braking of the rotor were necessary. Densities of tartrate gradient fractions were determined by weighing known volumes.

Plaque assays. Plaque assays were on confluent Vero cell monolayers grown in 5-cm-diameter petri dishes. Fractions from potassium tartrate gradients were slowly diluted in 10 mM sodium phosphate (pH 7.0) containing 140 mM NaCl (1:3) and, when required, treated with trypsin (50 µg/ml) for 15 min at 37°C. The reaction was stopped with soy bean trypsin inhibitor (100 µg/ml). Portions of the trypsinized samples were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis to verify that the Sendai virus F protein was cleaved while other proteins remained intact. For plaque assays, the trypsinized samples were slowly diluted as required with MEM containing 10 U each of penicillin and streptomycin per ml–10 mM HEPES–0.1% bovine serum albumin. Rapid dilution destroyed plaque formation. Virus adsorption was at 32°C for 1 h. Cells were overlaid with 0.2% agar in MEM supplemented with HEPES and albumin (2 ml per dish). Plaques were counted after 20 h at 32°C.

Analytical. Virions from the tartrate gradient fractions were pelleted by centrifugation (type JA-18.1 rotor [Beckman], 17,000 rpm for 1 h at 10°C). SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 10% gels (11). Samples were reduced and alkylated with an excess of iodoacetamide. Gels were stained by a silver-staining technique (18) or fixed and impregnated with En³Hance (New England Nuclear Corp., Boston, Mass.) for fluorography.

Radioactivity in gel bands was quantitated by excising the bands after fluorography. The bands were rehydrated in 150 µl of H₂O, after which 500 µl of Protosol (New England Nuclear) and 4 ml of Emulsifier Safe scintillation liquid (Packard Instrument Co., Inc., Groningen, Netherlands) were added. ³⁵S radioactivity was counted with a liquid scintillation counter after 24 h at 37°C.

For immunofluorescence microscopy of infected cells, a monoclonal antibody against Sendai virus hemagglutinin-neuraminidase (HN) (21) (clone 106) and affinity-purified rabbit anti-VSV G-protein antibodies were used (14). The immunocomplexes were visualized by immunofluorescence, using rhodamine-labeled anti-rabbit immunoglobulin G (IgG) and fluorescamine-conjugated anti-mouse IgG (13). The HN antibody did not cross-react with the G protein, nor did the anti-G-protein antibody cross-react with the HN protein.

Cell surface VSV G protein was immunologically quantitated by a ¹²⁵I-protein A (Amersham)-binding assay (22) on 24-well microdilution plates. Sendai virus F protein on the cell surface was similarly quantitated. A monoclonal F-protein antibody produced by Örvell and Grandien (20) (clone 802) was used, followed by incubation with an affinity-purified rabbit anti-mouse IgG (Jackson ImmunoResearch, Baltimore, Md.) and then with ¹²⁵I-protein A. Linearity of the assay over the range used was verified by using increasing amounts of double-infected, fixed cells scraped in suspension. Fifty-microliter reaction volumes were used in all incubations. Background binding was determined in the absence of the first antibody and was subtracted from the assay values.

RESULTS

Double infection with Sendai virus and VSV in Vero cells. VSV, a rhabdovirus, and Sendai virus, a member of the paramyxoviruses, were selected for double infections since their physical properties differ enough to facilitate separation. In Vero cells, the Sendai virus fusion protein is not cleaved and polykaryon formation peculiar to other paramyxoviruses is thus avoided. Superinfections were done with wt VSV or a G-protein-affected temperature-sensitive mutant, *ts045*.

To show that VSV grows in Sendai virus-infected cells, double-immunofluorescence staining was performed, using a monoclonal anti-HN antibody and polyclonal anti-VSV G-protein antibodies. Figure 1 demonstrates a double infection with Sendai virus and the mutant VSV *ts045* at 39°C. All cells were clearly stained with the antibodies against the spikes of both viruses. The stain intensity varied somewhat between cells. The HN shows smooth staining which marks the edges of the cells, suggesting surface localization. The G-protein stain is reticular, which is typical of internal localization, as expected for this mutant (37). When wt VSV was used to superinfect, the G protein and the HN both showed typical surface staining. All cells were double infected with both viruses (results not shown).

We found that VSV or the mutant *ts045* gradually switched off Sendai virus protein as well as cellular protein synthesis. Therefore, the virions were collected at an early time point (4 h) after superinfection to obtain optimal conditions for phenotypic mixing.

Particle purification. For biochemical analysis, the virions possessing the VSV genome must be separated from those possessing the Sendai virus genome. We used sedimentation through sucrose gradients followed by isopycnic centrifugation in potassium tartrate gradients. In the sucrose gradient,

FIG. 1. Immunofluorescent staining of Sendai virus HN protein and VSV G protein in cells dually infected with Sendai and *ts045* viruses. Vero cells were infected for 12 h with Sendai virus and then superinfected with *ts045*. After 4 h, cells were fixed, opened with Triton X-100, and processed for double indirect immunofluorescence by using a rabbit anti-VSV G-protein antibody and a mouse anti-Sendai virus HN monoclonal antibody. Primary labeled cells were stained with rhodamine-conjugated anti-rabbit IgG and fluorescamine-conjugated anti-mouse IgG. VSV G proteins are localized internally (left), while Sendai virus HN shows surface stain (right).

two distinct peaks were separated (data not shown). The material of the slowly sedimenting (light) peak on the sucrose gradient was found to band on the tartrate gradient as a single peak with a density of 1.128 g/cm³ when the mutant *ts045* was used to superinfect at 38 or 39°C. With Sendai virus and wt VSV double infection, the light peak of particles had a density of 1.133 g/cm³. These values correspond to the densities of spikeless *ts045* and wt VSV virions produced in single infections, respectively. The material from the fast-sedimenting (heavy) peak also banded as a single peak and showed a density of 1.140 g/cm³. Pure Sendai virions banded at 1.145 g/cm³. An example of a tartrate gradient centrifugation is shown in Fig. 2 for Sendai virus-*ts045* double infection at 38°C (semipermissive for G-protein transport). Polypeptide profiles of the proteins in the peaks on the tartrate gradient are also shown. The heavy peak seems to exhibit all the Sendai virus polypeptides and, in addition, the *ts045* polypeptides. The light peak contains the *ts045* polypeptides and an incomplete set of Sendai virus polypeptides, of which the F protein is the most prominent. Double-infection experiments with the mutant *ts045* at 39°C (nonpermissive temperature for G-protein transport) gave similar results, except that the yield of the putative *ts045* peak was lower and the G protein was totally lacking (results not shown).

Characterization of the purified virions. The viral polypeptide compositions of the light and heavy peaks were compared with those of pure *ts045* and Sendai virions. For this purpose, both the Sendai virus and the VSV *ts045* polypeptides were uniformly labeled, using continuous labeling. Figure 3 (lanes 1 and 2) shows the polypeptide profiles of both particles from continuously labeled, doubly infected cells grown at 39°C. Table 1 shows the quantitation of the polypeptide ratios. Such analyses indicate that the light particles exhibited *ts045* polypeptides in a normal ratio but predominantly spike components (HN and F) of the Sendai virus. This is consistent with the formation of phenotypically mixed *ts045* envelopes during double infection.

FIG. 2. Purification of phenotypically mixed Sendai and *ts045* virions on 16 to 22% potassium tartrate gradient. Virions were grown at 38°C and labeled with [³⁵S]methionine, using the prelabeling protocol. The first separation was by sedimentation in a 5 to 20% sucrose gradient (see Materials and Methods). Two peaks were observed. Three top fractions from each peak (total volume, 3 ml for each) were layered on a 16 to 22% potassium tartrate gradient and centrifuged for 90 min at 25,000 rpm (SW28 rotor [Beckman]) at 4°C. After fractionation, portions of fractions were counted for ³⁵S radioactivity. The radioactivity profiles on the tartrate gradient are shown for the fast-sedimenting (○) and the slowly sedimenting (●) particles. Positions of pure Sendai, wt VSV, and spikeless *ts045* virions are marked by arrows. SDS-PAGE analysis of the heavy peak (lane 1) shows all the Sendai virus and *ts045* polypeptides. A corresponding polypeptide profile of the light peak (lane 2) shows the Sendai virus glycoproteins (HN and F) and a small amount of the nucleoprotein (NP), in addition to the *ts045* polypeptides (L, G, N, and M). X and Y represent cellular proteins taken up by the *ts045* virions. Gels were stained with silver.

In contrast, the ratios of the Sendai virus and the *ts045* polypeptides of the heavy particles were both very similar to the polypeptide ratios of virions isolated from single infections. Such findings are consistent with the idea that *ts045* particles have adhered to Sendai virions. Furthermore, SDS-PAGE analyses indicated that the *ts045* proteins associated with the Sendai virus band peaked one fraction later on the tartrate gradient than the Sendai virus radioactivity (data not shown). Similar conclusions could be drawn for virions grown at 32 or 38°C in Sendai virus-*ts045* double infection. Furthermore, when virions were collected between 4 and 8 h after superinfection, essentially similar results were obtained for the light as well as the heavy peaks.

Additional support for our conclusions regarding the two types of mixed particles was obtained by mixing Sendai and *ts045* virions produced separately. Accordingly, viral progeny from Vero cells infected with either Sendai virus or VSV *ts045* were mixed and incubated for 1 h at 39°C, followed by gradient separation. SDS-PAGE shows that the light-density material from the tartrate gradient exhibited no Sendai virus polypeptides (Fig. 3, lane 4), while the heavy-density material showed a complete set of the polypeptides of both virions (Fig. 3, lane 3). We conclude that the Sendai virus-*ts045* adherent particles arose in the medium after budding. Because Sendai virions could not be separated from the

FIG. 3. Phenotypic mixing observed in dual infection but not by mixing of two viruses. Sendai virus and *ts045* were grown at 39°C in dual (2×10^7 cells; medium volume, 2 ml) and single (10^7 cells for each virus) infections. Media from single-infected cells (1 ml from each of the VSV- and Sendai virus-infected cell cultures) were mixed and incubated for 60 min at 39°C. Virus particles were then separated by sequential sucrose and tartrate gradient centrifugations (SW28.1 rotor). The top fraction of each peak was analyzed by SDS-PAGE. Gels were processed for fluorography. Lanes 1 and 2 show Sendai virus and *ts045* particles from the dual infection. Virions from single infections, after mixing and separation, are shown in lanes 3 (Sendai virus) and 4 (*ts045*). Sendai virus protein bands are not visible in the *ts045* virus which was mixed with the Sendai virus virions. Polypeptides P, HN, F, NP, and M refer to Sendai virus, and the others refer to VSV.

Sendai virus-VSV adducts, we could not analyze whether the Sendai virus adopts VSV G protein.

The Sendai virus nucleoprotein (NP) and traces of the matrix (M) protein were consistently seen in the phenotypic

TABLE 1. Viral polypeptide compositions in Sendai and *ts045* viruses grown in double- and single-infected cells at 39°C^a

Virus and polypeptide(s)	% of total polypeptides ^b after:		Single infection
	Double infection		
	Heavy peak	Light peak	
VSV <i>ts045</i>			
L	4.0 ± 0.1	4.3 ± 0.7	3.5 ± 0.2
N + NS	49.0 ± 1.6	46.8 ± 1.6	47.3 ± 0.3
M	47.1 ± 1.6	48.9 ± 0.8	49.3 ± 0.2
Sendai virus			
P	8.2 ± 0.5	ND ^c	8.1 ± 1.3
HN	14.0 ± 3.5	ND	11.4 ± 0.5
F	17.7 ± 1.1	ND	17.2 ± 0.3
NP	41.0 ± 1.0	ND	37.2 ± 0.5
M	19.1 ± 0.8	ND	26.1 ± 1.3
(HN + F)/NP ratio	0.77 ± 0.13	1.94 ± 0.06	0.77 ± 0.04

^a Infected cells were uniformly labeled with [³⁵S]methionine, virions were purified, and polypeptides were separated by SDS-PAGE. The polypeptide bands were excised and counted. The data are derived from two independent experiments. Means and interassay ranges are given.

^b For VSV *ts045*, total of L, N + NS, and M polypeptides; for Sendai virus, total of P, HN, F, NP, and M polypeptides.

^c ND, Not determined.

FIG. 4. Sendai virus F-protein-mediated VSV plaque formation. Virions harvested from cells (2×10^7 cells) double infected with Sendai virus and *ts045* at 39°C were separated by centrifugation in a 5 to 20% sucrose gradient followed by tartrate gradient centrifugation (SW28.1 rotor). Similarly, Sendai and *ts045* virions were produced in single infections at 39°C (10^7 cells for each virus). The media (1 ml plus 1 ml) were then mixed and incubated for 1 h at 39°C, and the virions were separated by sequential centrifugation. The material banding at the Sendai virus density as well as the material banding at the *ts045* density were then analyzed for VSV *ts045* infectivity by plaque formation at the permissive temperature (32°C). T, Trypsinized; N, nontrypsinized. The PFU per milliliter in the tartrate gradient fractions are shown. The mean (two determinations) and the range are shown.

ically mixed *ts045* virions. Trypsination of the virions did not totally abolish the Sendai virus nucleocapsid proteins in the *ts045* virions (data not shown). Thus, the question remains whether these proteins are inside the *ts045* particles or contaminating Sendai virions that possibly follow the *ts045* virions throughout the separation.

In addition to the *ts045* and Sendai virus components, the *ts045* virus exhibited two prominent bands, with apparent molecular weights of 23,000 and 125,000 (Fig. 2, lane 2). These polypeptides originate from vero cells and have been demonstrated before (12, 17).

Infectivity requires activation of the heterologous F protein. The Sendai virus glycoproteins (HN and F) in the VSV *ts045* envelope should mediate *ts045* infectivity, provided that the Sendai virus F protein is activated for fusion by proteolytic cleavage (9, 25). To analyze the Sendai virus spike-mediated infectivity, we used the mutant *ts045* produced at the non-permissive temperature (39°C) in dual infection. Such *ts045* particles cannot infect, since they lack G-protein spikes and the Sendai virus fusion protein is uncleaved. However, they contain the HN protein probably needed for attachment and should become infective when the Sendai virus fusogen is cleaved. Therefore, the virions were mildly trypsinized. Trypsin treatment of purified *ts045* virions from dual infection augmented the infectivity severalfold (Fig. 4). In contrast, *ts045* virions derived from single infection and incubated with Sendai virions did not show such an effect. The virions were left essentially noninfectious after trypsination.

FIG. 5. Quantitation of VSV G protein and Sendai virus F protein on the surface of double-infected cells. Cells were fixed with paraformaldehyde, and ¹²⁵I-protein A-binding assays were performed (see Materials and Methods). To determine background binding, mock labelings were done by leaving out the anti-spike antibodies. The background was 2 to 3% of the total (200,000 cpm). The mean (two determinations) and the range are shown.

This result suggests that the infective particles are phenotypically mixed and that their fusion to cells is mediated by the Sendai virus F protein after trypsin cleavage activation. Note that plaque assays were done at 32°C, the permissive temperature for *ts045*, and in the absence of trypsin. Under such conditions, *ts045* plaques develop but Sendai virus plaques do not.

When the Sendai virus-*ts045* adducts were similarly analyzed, a marked increase in infectivity was also seen after trypsin treatment. This occurred with the sample originating from double infection as well as with that obtained by mixing Sendai and *ts045* virions (Fig. 4). We conclude that Sendai virus-*ts045* adducts behave also in plaque assays as phenotypically mixed particles (pseudotypes).

Extent of phenotypic mixing. The previous analyses are consistent with the conclusion that VSV incorporates foreign spikes. To analyze whether the Sendai virus glycoproteins could replace an essential amount of the G protein in VSV, we used the mutant *ts045*. This mutant has a transport block of the G protein at 39°C (10, 37). At lower temperatures, the G protein gradually moves to the cell surface. Thus, it should be possible to regulate the Sendai virus glycoproteins/G-protein ratio on the cell surface by regulating the incubation temperature.

Accordingly, double infections were done at 32, 38, and 39°C. Immunological quantitation showed that the amount of Sendai virus F protein on the cell surface remained fairly constant over the 32 to 39°C temperature range, while that of the G protein dropped radically with increasing temperature (Fig. 5). Thus, there should be more favorable conditions at 39°C for phenotypic mixing than at the lower temperatures. However, this was not the case. Figure 6 shows a typical SDS-PAGE profile of the mixed phenotypes produced at each temperature. To quantitate the extent of phenotypic mixing, the gel bands were excised and counted for ³⁵S-radioactivity (Table 2). It is evident that the incorporation of Sendai virus spikes into the VSV *ts045* membrane did not differ significantly when budding occurred from a plasma membrane containing high or low G-protein/Sendai virus glycoprotein ratios. We found that under all conditions, 104 to 125 F polypeptides and 40 to 77 HN polypeptides were

FIG. 6. Phenotypically mixed *ts045* virions produced at different temperatures. Sendai virus-infected cells were superinfected with the *ts045* mutant and, after virus adsorption, were grown at the indicated temperatures. Continuous labeling with [³⁵S]methionine was used. The *ts045* virions were purified by sequential sucrose and tartrate gradient centrifugations and subjected to SDS-PAGE. Polypeptide bands (as described in the legend to Fig. 2 and 3) were excised and counted for radioactivity to produce the data for Table 2.

incorporated into a *ts045* virion, altogether corresponding to 12 to 16% of the number of G proteins in normal VSV. Furthermore, we found that wt VSV also acquired essentially similar amounts of Sendai virus spikes when grown in double infection (data not shown). Taken together, these results suggest that the heterologous glycoproteins did not compete for G-protein sites in the budding virion. The results do not support a selective, VSV protein-mediated inclusion mechanism.

When *ts045* particle production was monitored, a significant drop was noted during double infection when the temperature was raised. At 39°C, the yield of *ts045* particles was about 30% of that at 38°C and 5 to 10% of that at 32°C. Since there were roughly equal amounts of the Sendai virus glycoproteins on the cell surface at each temperature, while the amount of the G protein decreased with temperature (Fig. 5), we conclude that the Sendai virus spikes are not able to compensate for the G protein in budding.

DISCUSSION

It has been suggested that viral glycoproteins other than the G protein can be used to form the bullet-shaped VSV particle (16). While some authors hold the view that the G protein is not essential for VSV formation (32), others have suggested that it is needed for the nucleation of budding (33). In this work, we have approached the question of efficiency of the phenomenon of phenotypic mixing. The temperature-sensitive VSV mutant *ts045* was used as a tool to modify the ratio of homologous to heterologous spikes on the cell surface. Our results show that there was no increase in the number of the heterologous spikes in *ts045* virions when the ratio of Sendai virus glycoproteins to the G protein was increased by raising the temperature. Quantitative analysis

TABLE 2. Quantitation of Sendai virus glycopolypeptides in phenotypically mixed *ts045* virions from double infections^a

Temp and polypeptide	dpm ^b	% of G in wt VSV ^c	No. of polypeptides in <i>ts045</i> virion ^c
32°C			
L + N + NS + M	11,771		
G	1,341	59.8 ± 3.0	721 ± 36
HN	130	6.4 ± 1.6	77 ± 19
F	175	9.5 ± 2.3	115 ± 28
38°C			
L + N + NS + M	9,981		
G	314	16.5 ± 1.4	199 ± 17
HN	103	6.0 ± 1.9	72 ± 23
F	161	10.3 ± 2.0	125 ± 24
39°C			
L + N + NS + M	13,039		
G	0	0	0
HN	75	3.3 ± 2.0	40 ± 24
F	176	8.7 ± 1.2	104 ± 14

^a Bands were excised (Fig. 6) and counted for ³⁵S radioactivity. The number of methionine residues in the Sendai virus glycopolypeptides (9 in the F and 10 in the HN polypeptides) (27) as well as in the wt VSV and *ts045* G proteins (11 residues) (7, 23) are known. From the known amount of ³⁵S radioactivity in the G protein of wt VSV (16%) and the known number of methionine residues in the viral polypeptides, we calculated the theoretical amount (percent) of G protein in wt VSV that each glycoprotein represented. Assuming that there are 1,205 G-protein monomers in a VSV virion (28), the number of each glycopolypeptide in a *ts045* virion was calculated.

^b Mean disintegrations per minute from two independent experiments are given.

^c Mean and the range are given.

of the foreign glycoproteins in the mutant *ts045* particles produced in double infections at different temperatures showed that only 9 to 10% of F protein and 3 to 6% of HN protein could be incorporated relative to the theoretical number of G-protein spikes (Table 2). Thus, there seems to be a limit for the incorporation of foreign spikes into the *ts045* envelope. Furthermore, the yield of *ts045* physical particles decreased considerably during double infection at 39°C, when there is a shortage of G protein, compared with the yield at 38°C. Such results emphasize the role of the G protein in generating viral particles. The presence of Sendai virus glycoproteins did not compensate for the G protein to increase the yield of physical *ts045* particles. The Sendai virus glycoprotein could, however, rescue the infectivity of *ts045* particles (Fig. 4). This result agrees with the study of Weiss and Bennett (32) in which spikeless *ts045* particles were rendered infective by retroviral spikes. In their study, however, the production of physical particles was not analyzed.

It has been shown that the mutant *ts045* buds with intact G proteins which are then cleaved, leaving the G-protein C-terminal fragments in the virus membrane (17). This fact and the finding in this study that the heterologous spikes could not compete for G-protein sites nor replace the G protein in budding suggests that VSV budding requires a full or a nearly full number of G-protein spikes. During budding of the *ts045* virus at 39°C, the C-terminal fragments possibly replace part of the G protein (3). Since the yield of physical particles dropped when there was a lack of G protein on the plasma membrane, we conclude that high levels of such fragments are not used for virus formation. Such fragments are probably not excessively available on the plasma mem-

brane at the nonpermissive temperature, since the G protein accumulates in the endoplasmic reticulum.

We suspect that the pseudotype VSV particles which have been documented elsewhere (32, 33, 36) are not fully covered with heterologous spikes. Rather, only a small fraction of the theoretical spike protein positions are taken up by foreign glycoproteins, the rest being G-protein fragments. The relatively low and constant amounts of Sendai virus glycoproteins that we found in the *ts045* particles at different temperatures suggests that inclusion of foreign spikes could occur randomly because of the abundance of Sendai virus glycoproteins. It remains to be discriminated whether the foreign glycoproteins really replace G proteins or occupy spaces between the homologous spikes or spike C-terminal fragments. Remarkably, the rather low number of Sendai virus spikes made the *ts045* virions infectious. Presumably, the foreign spikes are arranged as patches to fulfill the requirement of critical spike density needed for fusion (6, 29). One interpretation for phenotypic mixing is that cellular vesicles containing a set of Sendai virus proteins bind and fuse to VSV particles. This would explain why the internal Sendai virus proteins were found in the phenotypically mixed particles. However, the results from in vitro mixing of Sendai virus and *ts045* harvests (Fig. 4) do not support this view. Furthermore, immunoelectron microscopy studies with other double-infection systems have not revealed vesicles attached to VSV but instead normal VSV particles carrying heterologous spikes (1, 2, 4).

Two cellular polypeptides were found in the *ts045* virions purified from double-infected cells (Fig. 2). These polypeptides are also present in *ts045* virions produced in single infection (17). Presumably, they are identical to those described by Lodish and Porter (12). Their presence in the spikeless *ts045* virions does not measurably reduce the number of G-protein C-terminal fragments found in these particles, compared with that of the wt VSV (17). Thus, it seems likely that during double infection they occupy a significant number of neither G-protein positions nor G-fragment positions. In this case, the identity of these cell-derived proteins is not known. In several other cases, the cellular proteins in VSV have been shown to be of retroviral origin (24).

We were able to separate VSV virions from Sendai virions but not vice versa because VSV and Sendai virus aggregated, forming heterodimers or higher oligomers. The physical properties of such adducts resemble those of the parent Sendai virus so closely that separation was not successful. It is likely that the attachment between Sendai virus and VSV occurs through the HN protein and the sialic acid in the G protein. This phenomenon has been shown for the related influenza virus hemagglutinin (35). Why the spikeless *ts045* virions also were adsorbed to Sendai virions remains obscure. One possibility is that there are some G-protein spikes left in the *ts045* virions, sufficient to mediate the attachment to the HN.

Plaque analysis of the *ts045*-Sendai virus adducts showed that they behaved functionally as pseudotypes. Plaques were seen only when the Sendai virus F protein was activated by trypsin. Our explanation is that the Sendai virion fuses to both the spikeless *ts045* envelope and the cell membrane. Thus, both genomes arrive in the cytoplasm. Because VSV replicates more rapidly than Sendai virus, only VSV progeny are seen. Probably, such apparent pseudotypes would be inactivated by Sendai virus antibodies but not by VSV antibodies. Likewise, we presume that wt-VSV-Sendai virus adducts behave as double-neutralizable particles. In an

earlier study, Zavada and Rosenbergova (35) showed that fowl plague virus and VSV form similar adducts, which are double neutralizable. In their study as well as ours, such heterodimeric viral adducts arose by *in vitro* mixing and during double infection. This phenomenon is likely to be more common between different viruses. For example, we have found it very difficult to separate VSV from Semliki Forest virus because of Semliki Forest virus-VSV adducts (unpublished observations). This facet of phenotypic mixing, however, has been overlooked in several studies.

In conclusion, our study shows that VSV assembles its own glycoprotein more efficiently than the Sendai virus glycoproteins. There is only a limited amount of space for foreign viral glycoproteins in the VSV envelope.

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